Acute inflammation and infection maintain circulating phospholipid levels and enhance lipopolysaccharide binding to plasma lipoproteins

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Abstract Circulating lipoproteins are thought to play an important role in the detoxification of lipopolysaccharide (LPS) by binding the bioactive lipid A portion of LPS to the lipoprotein surface. It has been assumed that hypocholesterolemia contributes to inflammation during critical illness by impairing LPS neutralization. We tested whether critical illness impaired LPS binding to lipoproteins and found, to the contrary, that LPS binding was enhanced and that LPS binding to the lipoprotein classes correlated with their phospholipid content. Whereas low serum cholesterol was almost entirely due to the loss of esterified cholesterol (a lipoprotein core component), phospholipids (the major lipoprotein surface lipid) were maintained at near normal levels and were increased in a hypertriglyceridemic subset of septic patients. The levels of phospholipids found in the LDL and VLDL fractions varied inversely with those in the HDL fraction, and LPS bound predominantly to lipoproteins in the LDL and VLDL fractions when HDL levels were low. Lipoproteins isolated from the serum of septic patients neutralized the bioactivity of the LPS that had bound to them. Our results show that the host response to acute inflammation and infection tends to maintain lipoprotein phospholipid levels and that, despite hypocholesterolemia and reduced HDL levels, circulating lipoproteins maintain their ability to bind and neutralize an important bacterial agonist, LPS.—Kitchens, R. L., P. A. Thompson, R. S. Munford, and G. E. O'Keefe. **Acute inflammation and infection maintain circulating phospholipid levels and enhance lipopolysaccharide binding to plasma lipoproteins. *J. Lipid Res.* 2003.** 44: **2339–2348.**

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Lipopolysaccharide (LPS) (also called endotoxin), one of the most potent of the known bacterial agonists, is released from the outer membranes of gram-negative bacteria and is thought to be an important trigger for the host response known as sepsis. In addition to their roles in cholesterol and lipid transport, plasma lipoproteins provide an important host mechanism for controlling responses to LPS (1). Lipoproteins bind the bioactive lipid A portion of the molecule and prevent it from stimulating monocytes, macrophages, and other LPS-responsive cells. Lipoprotein-bound LPS is cleared from the circulation principally by the liver (2), where the LPS may then be excreted into the bile (3). In numerous in vivo experimental models, raising plasma lipoprotein levels has decreased the stimulatory effects of LPS and increased host survival during endotoxemia (4, 5) or gram-negative bacterial infection (6). Conversely, lowering lipoprotein levels rendered animals more susceptible to LPS-induced lethality (1).

Extensive changes in plasma lipoproteins occur during the body's acute-phase response to injury or infection (7). For many years, hypocholesterolemia has been a consistent finding in humans with infection or critical illness (7, 8). Several reports have shown strong correlations between low plasma cholesterol and mortality in critically ill or infected patients (8–10), and some investigators have hypothesized that low lipoprotein cholesterol levels impair the host's ability to bind and neutralize LPS, so that more LPS is available to induce harmful inflammation (8, 10–12). Low cholesterol levels are sometimes accompanied by hypertriglyceridemia and elevated VLDL levels (7. 13). Hypertriglyceridemia is generally thought to benefit the host in various ways [e.g., by providing nutrients to cells involved in the immune response and tissue repair and by neutralizing certain microorganisms and their toxins (14)]. No detailed analysis of LPS-lipoprotein interactions in the serum of critically ill patients has been reported, and it is uncertain how hypocholesterolemia or

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hypertriglyceridemia might affect innate immune responses and inflammation in these patients.

Phospholipids are the most abundant lipid components of the surface domain of lipoprotein particles (15), and several lines of evidence suggest that lipoprotein phospholipids are the most important determinants of LPS-lipoprotein binding: Parker and coworkers (16) showed that the ability of the various lipoprotein classes to neutralize the bioactivity of LPS depends upon their phospholipid content and not upon their content of cholesterol or triglycerides; LPS binding protein (LBP) can transfer LPS into phospholipid vesicles (17); and artificial phospholipid-rich particles (4, 18) and emulsions (19) are effective LPS scavengers. In therapeutic trials, increasing the phospholipid content of circulating lipoproteins was associated with increasing protection against the adverse physiologic effects of LPS in rabbits (20) and against mortality in a model of porcine septic peritonitis (19).

HDL is the most abundant lipoprotein in terms of particle number and surface area, and it contains the majority of the circulating phospholipids in normal human plasma (11, 15, 21). When LPS is added to plasma or serum, the majority of the lipoprotein-bound LPS is found in the HDL fraction, whereas most of the remainder is found in LDL (22–24). In septic patients, HDL levels decline more than those of any other lipoprotein class (25). LDL cholesterol levels also fall sharply, raising the possibility that LPS binding to both HDL and LDL could be impaired. In contrast, the VLDL and chylomicron (CM) fractions of normal serum bind very little LPS, even when VLDL and CM levels are elevated, as in healthy postprandial human subjects (26). LPS binding to VLDL was only modestly increased (<2-fold) in the blood of subjects with type IV hyperlipidemia; in these specimens, HDL remained the dominant LPS acceptor despite severe hypertriglyceridemia and high VLDL levels (27). Although these results suggest that VLDL may play a minor role in LPS neutralization, infusion of exogenous VLDL or CM can protect rodents from LPS-induced lethality and can prevent septic death in a model of infectious peritonitis (6). The role that these triglyceride-rich particles play in LPS neutralization is thus uncertain.

To understand the impact of critical illness and infection on the ability of endogenous human lipoproteins to bind LPS, we measured the binding of radiolabeled LPS to lipoproteins in the serum of critically ill patients, many of whom were experiencing severe sepsis or septic shock, and compared these values with those obtained from a group of healthy volunteers.

MATERIALS AND METHODS

LPS and reagents

Biosynthetically labeled *Escherichia coli* LCD25 ([^{3}H]LPS, Ra chemotype [1.5×10^6 dpm/μg]) (28) was diluted in HEPES-buffered saline that contained 0.1 mM EDTA and 0.3 mg/ml of BSA and sonicated to disperse large LPS aggregates as described (29). All other reagents were from Sigma Aldrich (St. Louis, MO) unless stated otherwise.

Experimental subjects

Serum samples were obtained from 41 critically ill patients and 17 healthy control subjects (median ages 41 and 37 years, respectively). Thirty-one of the patients had sustained blunt trauma or severe burn injury, and the remaining 10 patients had various other illnesses. Cholesterol and lipid analyses were performed on all subjects, and complete analyses of fractionated lipoproteins were performed on a representative group of 19 of these patients and seven controls. In some patients, two to four serum samples were taken from the same individual on different days, and these were included in the analysis if significant changes in the patients' clinical condition had occurred. Clinical classifications were made according to established criteria (30). All of the samples were from patients who met criteria for the systemic inflammatory response syndrome (SIRS). Patients with no evidence of infection were classified as "SIRS." Patients who had confirmed bacterial or fungal infections were classified as "sepsis," and sepsis patients with organ dysfunction, hypoperfusion, hypotension, or shock were classified as "severe sepsis."

Serum was prepared by centrifugation of clotted venous blood, and it was aliquoted and stored at −70°C. The samples were obtained by informed consent with the approval of the Institutional Review Board of UT Southwestern Medical Center.

Isolation and fractionation of LPS-lipoprotein complexes

Undiluted serum (0.5 ml) was mixed with 26 μl of a protease inhibitor cocktail to give final concentrations of 1 μg/ml aprotinin, 1 mM benzamidine, 0.25 mM PMSF, and 20 mM HEPES buffer (pH 7.4). Two and a half microliters of [^{3}H]LPS (50 ng LPS/ml final) was then added, the mixture was incubated for 10 min or 60 min at 37°C, and the reaction was stopped by placing the sample on ice. The total lipoprotein fraction of the serum was then isolated by ultracentrifugal flotation in KBr ($d < 1.21$ gm/ml) as described (23) in the presence of the same protease inhibitors and 3 mM EDTA. Free [^{3}H]LPS and [^{3}H]LPS-protein complexes did not float with LPS-lipoprotein complexes. In experiments not shown, the protease inhibitors did not influence the binding or distribution of [^{3}H]LPS in the lipoprotein classes.

The total lipoprotein fraction (0.5 ml) was separated into the major lipoprotein classes on a Superose 6 HR 10/30 column using an AKTAexplorer 10 chromatography system (Amersham Pharmacia Biotech, Piscataway, NJ). The column buffer [50 mM Tris (Cl^-) (pH 7.4), 150 mM NaCl, 0.25 mM EDTA, and 0.02% sodium azide] was run at a flow rate of 0.3 ml/min, and 0.5 ml fractions were collected. The recovery of [^{3}H]LPS in the column fractions was 95 ± 6% (mean ± SD, n = 58) of the material injected onto the column. The presence of LPS in the lipoprotein particles did not alter their mobility on the column; this may be due to the small molecular size of the LPS monomers (~4 kDa) and to the low LPS-lipoprotein ratio. The lipoprotein-bound [^{3}H]LPS separated in the same positions as the lipoprotein cholesterol and phospholipids. Ultracentrifugal flotation of the LPS-lipoprotein complexes prior to Superose 6 chromatography was required to remove free LPS and protein-bound LPS, both of which had unpredictable mobilities on the column.

To identify the lipoproteins in the column fractions, apolipoproteins were measured by ELISA either in individual fractions or in pooled fractions that constituted each peak. The lipoproteins separated into three distinct peaks in samples from all controls and from almost all of the patients. Peak I, which contained VLDL and CM, had substantial amounts of triglycerides and nearly one-half of the total apolipoprotein E (apoE). Ratios of triglycerides to phospholipids in peak I did not exceed 2.2, suggesting that these particles were predominantly VLDLs and not CMs, which normally contain much higher ratios (~12) (15).

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We cannot rule out the possibility that this peak also contains large phospholipid-rich particles such as lipoprotein X (31). Peak II contained LDL, as evidenced by the predominance of apoB [81 ± 12% of the total apoB (n = 31) in the patients and 85 ± 6% (n = 7) in the controls]. Peak III contained all of the HDL and no LDL, as shown by the presence of over 95% of the total apoA-I and less than 5% of the total apoB in both control and patient samples. Serum amyloid A (SAA), which becomes the major HDL apolipoprotein during the acute-phase response [as reviewed in ref. (11)], was elevated in the patient serum [mean = 3.4 mg/ml (range 0.39–10.8); n = 43]; as expected, we found almost all of the SAA (87 ± 17%; n = 31) in the HDL peak. In a subset of hypertriglyceridemic patients, however, both SAA and apoB were increased substantially in the VLDL peak, in keeping with the work of others (32) who showed by a density fractionation method that apoB and SAA can become major constituents of VLDL at the height of the acute-phase response. We did not identify a discrete peak for IDL, which is normally intermediate between LDL and VLDL in both density and size. Therefore, peaks I and II may both contain IDL. Lipoprotein [a] (Lp[a]) separated on the leading edge of the LDL peak in normal plasma (data not shown); circulating Lp[a] has been reported to be markedly reduced in patients with burn injury and sepsis (33).

Lipoprotein assays

Cholesterol and lipids were measured colorimetrically in a microtiter format. Infinity™ reagent sets for total (esterified and unesterified) cholesterol and triglycerides were from Sigma-Aldrich. The Phospholipids B™ assay kit (Wako Chemical Co., Richmond, VA) detected both phosphatidylcholine and sphingomyelin, which constitute over 90% of the lipoprotein phospholipids. Sphingomyelin was measured in total lipid extracts of serum by a colorimetric assay using sphingomyelinase as described previously (34), with brain sphingomyelin as the standard. Phosphatidylcholine was undetectable in this assay. Total phospholipids were measured in both whole serum and the lipid extracts using the Phospholipids B™ assay to determine lipid recovery in the extracts. Free (unesterified) cholesterol was measured in serum using the assay kit from Wako Chemical Co., and esterified cholesterol was calculated by subtracting free from total cholesterol. Total serum apoA-I, apoB, and apoE were measured turbidimetrically using assay kits from Sigma-Aldrich, DiaSorin (Stillwater, MN), and Wako Chemical Co., respectively. To measure apolipoproteins in the column fractions, ELISA assays were performed using antibodies from BioDesign International (Saco, ME). The monoclonal capture antibodies were clones 1C5 (H61531M) for apoA-I, 4C11 (H61527M) for apoB, and 1H4 (H11004M) for apoE. Biotinylated goat antibodies were used for detection (K45252G, K34003G, and K74180B, respectively). For SAA, a matched antibody pair (C-SAA1 and T-SAA2-H) was obtained from YES Biotechnology Laboratories, Ltd. (Ontario, Canada). Streptavidin-conjugated horseradish peroxidase (Jackson Immunoresearch, West Grove, PA) and 3,3′,5,5′-tetramethylbenzidine substrate reagent (BD Pharmingen, Chicago, IL) were used for detection.

LPS bioactivity assay

Free [^{3}H]LPS or dialyzed [^{3}H]LPS-lipoprotein complexes were incubated with adherent normal human monocytes in 1 ml RPMI 1640 containing heat-inactivated serum (10% FCS and 1% human serum) for 6 h at 37°C in a 5% CO_2 atmosphere. Tumor necrosis factor (TNF)-α and interleukin 6 (IL-6) were measured in the culture supernatants by ELISA using DuoSet™ reagent sets from BD Pharmingen.

Statistics

Data sets that were segregated according to clinical classification were analyzed by ANOVA with Dunnett's multiple comparison test, and sets of X-Y data points were analyzed by linear regression or nonlinear exponential regression using Prism 3.0 (GraphPad Software, Inc., San Diego, CA). The analyses included some data points that were derived from the same patient at a different clinical stage of illness (see above). Removing these replicate samples did not alter the P values in ANOVA and regression analyses, and r values changed very little (data not shown).

RESULTS

Serum lipid profiles in critically ill patients

The patients in our study can be grouped according to two general lipid profiles: *a*) hypocholesterolemia without hypertriglyceridemia and *b*) hypocholesterolemia with varying degrees of hypertriglyceridemia. As shown in **Fig. 1A**, every clinical group showed marked hypocholesterolemia, which was due almost entirely to reduced levels of cholesterol esters (Fig. 1B). In contrast, free cholesterol was not significantly reduced in the patient population and was increased in a subset of patients with severe sepsis (Fig. 1C). Total phospholipid levels, which correlated strongly with those of free cholesterol ($r = 0.91$; $P < 0.0001$; data not shown), declined slightly in SIRS patients and increased in a subset of severe sepsis patients (Fig. 1D). Changes in total phospholipid levels were due mainly to changes in phosphatidylcholine levels. Sphingomyelin constituted a small fraction (5–14%) of the total phospholipids, and sphingomyelin levels were slightly decreased [15 ± 3.8 mg/dl (mean ± SD), n = 17] in the patient serum compared with those of normal serum (23 ± 2.8 mg/dl, n = 7) ($P = 0.0001$; data not shown).

A subset of patients had hypertriglyceridemia (Fig. 1E), defined here as a serum triglyceride level above 200 mg/dl. Hypertriglyceridemia occurred regardless of whether the patients received nutritional supplementation (data not shown), and these patients also had elevated levels of phospholipids, free cholesterol, and VLDL (see below). In the patient population, total serum phospholipids ($r = 0.90$; $P < 0.0001$) and free cholesterol ($r = 0.89$; $P < 0.0001$) increased coordinately with triglycerides, whereas cholesterol ester levels were inversely related to those of triglycerides ($r = -0.48$; $P = 0.0043$).

HDL phospholipids vary inversely with VLDL and LDL phospholipids

Analysis of the major lipoprotein classes showed that HDL levels decreased sharply in the patient population. As shown in **Table 1**, the greatest decline in HDL cholesterol and phospholipid levels (87% and 71%, respectively) occurred during severe sepsis. Although LDL cholesterol also decreased by 55% in the patient serum, phospholipid levels in the LDL and VLDL fractions did not decline at all. As shown in **Fig. 2**, LDL and VLDL phospholipids increased as HDL declined to very low levels, suggesting a compensatory mechanism for the loss of

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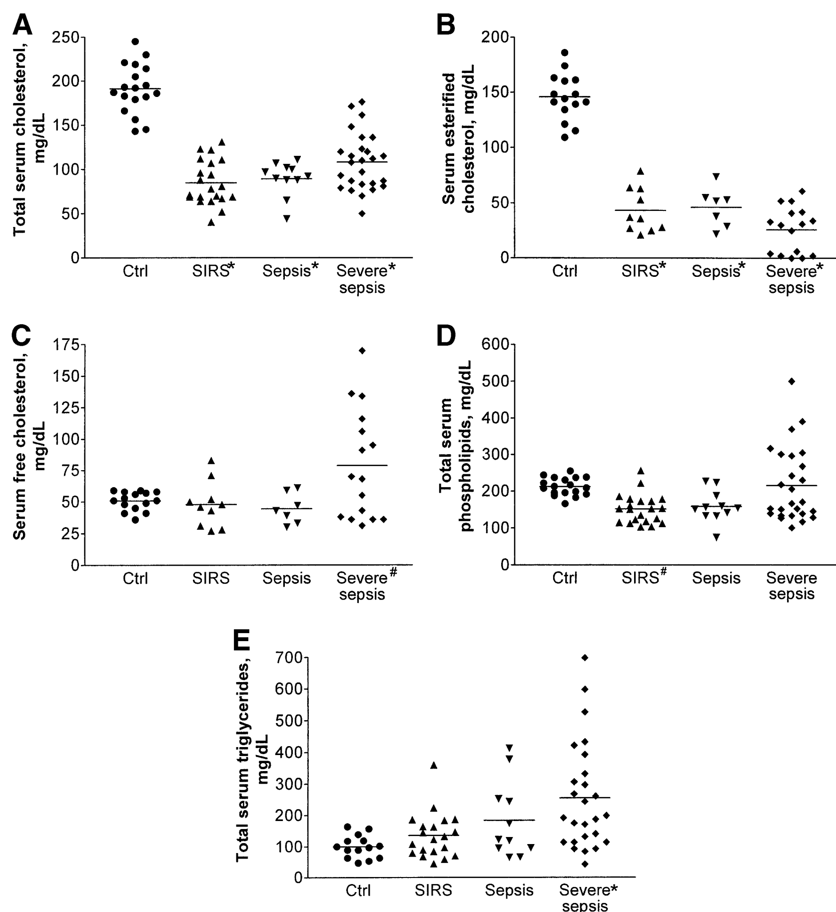

Fig. 1. Total serum cholesterol and lipids in healthy controls and critically ill patients. Measurements of total serum cholesterol (esterified and unesterified) (A), esterified cholesterol (B), free (unesterified) cholesterol (C), phospholipids (D), and triglycerides (E) are shown for healthy controls (Ctrl) and patients according to clinical classification. Significant differences from controls are signified by * $P < 0.01$ and # $P < 0.05$.

HDL phospholipids. These inverse relationships were nonlinear, mainly due to the fact that HDL levels were often low in the absence of an increase in VLDL and LDL. VLDL cholesterol also increased with decreasing HDL levels, whereas LDL cholesterol remained low (data not shown). Increases in VLDL ($r = 0.89$; $P < 0.0001$) and LDL phospholipids ($r = 0.63$; $P < 0.0001$) were related to increased triglycerides.

LPS-lipoprotein binding is enhanced during critical illness

To determine the relative rates of LPS binding to plasma lipoproteins, we measured the extent of [^{3}H]LPS binding to lipoproteins during an incubation time (10 min) at which submaximal binding occurred. We found that the mean percentage of lipoprotein-bound [^{3}H]LPS was higher in the patient samples [50 ± 6.8% (mean ± SD), n = 31] than in controls (35 ± 3.3%, n = 10, $P <$ 0.001). When the incubations were extended to 60 min, the lipoprotein-bound [^{3}H]LPS in the patient samples (64 ± 7.0%, n = 6) was still higher than that in the controls (52 ± 3.1%, n = 3, $P < 0.05$). Despite low cholesterol levels, the rates of LPS binding to lipoproteins were significantly increased in all clinical groups (**Fig. 3A**).

Because LBP is the major serum protein that transfers LPS to lipoproteins (22), we next measured serum LBP concentrations to determine if elevated levels of LBP might be responsible for the increased rates of LPS-lipoprotein binding in the patient serum. Although the mean LBP concentration in the patient samples (28 ± 17 μg/ml; n = 36) was elevated ~10-fold above levels in controls, regression analysis showed no correlation between the LBP concentration and the rate of LPS binding to lipoproteins ($r = 0.076$, $P = 0.68$). In contrast, certain changes in serum lipoproteins showed highly significant correlations with LPS binding to the lipoproteins. The ratio of phospholipids to total cholesterol was elevated to varying degrees in all clinical groups (Table 1), and this ratio correlated most strongly with LPS binding to lipoproteins (Fig. 3B). We looked for other correlations within the patient population (controls were excluded to increase confidence) and found that in addition to the phospholipid-to-total cholesterol ratio, total serum apoE, apoB, and LDL phospholipids each correlated positively ($P < 0.01$) with LPS binding to the total lipoprotein fraction. These results raise the possibility that changes in lipoprotein composition may be responsible for the observed enhancement of LPS binding.

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TABLE 1. Phospholipid, total cholesterol, and [^{3}H]lipopolysaccharide distribution in the major lipoprotein classes in critically ill patients

	Control (n = 7)	SIRS (n = 11)	Sepsis (n = 8)	Severe Sepsis (n = 16)
HDL				
Phospholipids	130 ± 22 (85–155)	61 ± 26 (27–99)[a]	62 ± 18 (32–88)[a]	38 ± 19 (14–73)[a]
Cholesterol	66 ± 9 (54–79)	21 ± 12 (8–43)[a]	15 ± 8.4 (5–30)[a]	8.4 ± 7.5 (1–28)[a]
PL-cholesterol ratio	2.0 ± 0.2 (1.6–2.2)	3.3 ± 0.8 (2.2–4.2)	5.0 ± 3.3 (2.9–12.3)	7.0 ± 4.9 (2.4–27)[a]
[^{3}H]LPS	61 ± 4.7% (53–68)	60 ± 14% (38–75)	57 ± 12% (37–69)	38 ± 17% (13–62)[a]
LDL				
Phospholipids	74 ± 16 (57–96)	72 ± 34 (32–125)	74 ± 20 (55–111)	100 ± 41 (46–177)
Cholesterol	113 ± 21 (89–138)	51 ± 17 (29–73)[a]	58 ± 13 (35–70)[a]	52 ± 15 (28–84)[a]
PL-cholesterol ratio	0.66 ± 0.05 (0.57–0.7)	1.4 ± 0.4 (0.8–1.9)	1.3 ± 0.4 (0.9–1.9)	2.0 ± 0.8 (1.0–3.8)[a]
[^{3}H]LPS	34 ± 5.6% (26–43)	30 ± 9.4% (19–47)	33 ± 9.2% (23–50)	39 ± 7.8% (29–53)
VLDL				
Phospholipids	14 ± 8.4 (4–26)	30 ± 29 (9–96)	28 ± 31 (0–92)	102 ± 97 (2–340)[b]
Cholesterol	17 ± 11 (8–39)	18 ± 13 (8–49)	18 ± 18 (1–56)	47 ± 40 (1–134)
PL-cholesterol ratio	0.82 ± 0.22 (0.5–1.2)	1.5 ± 0.5 (0.8–2.3)[b]	1.3 ± 0.7 (0–1.9)	1.9 ± 0.42 (1.0–2.5)[a]
[^{3}H]LPS	4.9 ± 1.8% (3–8)	9.8 ± 6.7% (3–25)	10 ± 8.9% (3–29)	23 ± 16% (2–58)[a]

LPS, lipopolysaccharide; SIRS, systemic inflammatory response syndrome. Total serum lipoproteins ($d < 1.21$ g/ml) were isolated and fractionated by Superose 6 chromatography. The total phospholipid or cholesterol content of each lipoprotein class (expressed as mg/dl of serum) was determined by multiplying the percentage of the total phospholipids or cholesterol found in each peak (I, VLDL; II, LDL; and III, HDL as shown in Fig. 4) by the concentrations of total serum phospholipids or cholesterol, respectively. The ratio of phospholipids to cholesterol was measured in each individual sample, and the averages of those measurements are shown. The [^{3}H]LPS distribution is shown as the percentage of total lipoprotein-bound [^{3}H]LPS found in each fraction as described in Fig. 3. The data are shown as mean ± SD (range). Subjects were segregated by clinical classification of critically ill patients.

[a] Significant differences from the control group, $P < 0.01$.

[b] Significant differences from the control group, $P < 0.05$.

LPS binds predominantly to HDL in normotriglyceridemic serum

We next measured LPS binding to the major lipoprotein classes by Superose 6 column chromatography. Representative chromatograms of lipoproteins from healthy volunteers and septic patients are shown in **Fig. 4**. The mean percentages of the total lipoprotein-bound [^{3}H]LPS found in each lipoprotein fraction are shown in Table 1 for control and patient serum. As expected (23), in normal serum we found the majority of the lipoprotein-bound [^{3}H]LPS in the HDL fraction [61 ± 5% (mean ± SD), n = 7], with most of the remainder in LDL (34 ± 6%) and a small amount in VLDL (5 ± 2%) (Table 1, Fig. 4A, B, and **Fig. 5**). Despite low HDL levels in the patient samples (Table 1), HDL was the dominant acceptor lipoprotein in normotriglyceridemic serum, as shown in samples with low VLDL levels (Fig. 5A).

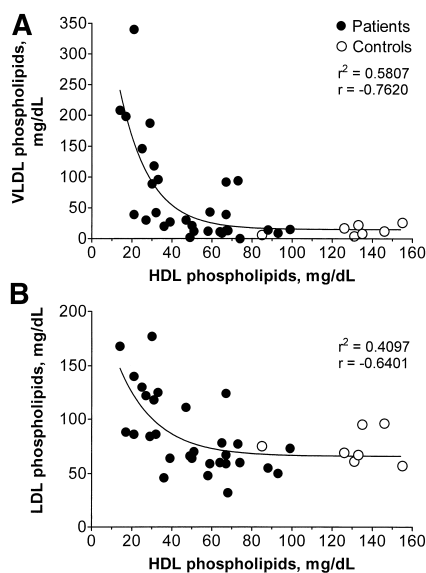

Fig. 2. VLDL phospholipids (A) and LDL phospholipids (B) vary inversely with HDL phospholipids. Lipoproteins from controls and critically ill patients were analyzed as described in Fig. 4, and the total phospholipid content of each lipoprotein class (mg/dl of serum) was determined as described in Table 1. The data were analyzed by exponential regression.

LPS binds predominantly to LDL and VLDL in hypertriglyceridemic serum

LPS binding to VLDL ([^{3}H]LPS-VLDL) increased linearly with increasing VLDL phospholipids in hypertriglyceridemic serum, and there was an inverse relationship between [^{3}H]LPS-VLDL and [^{3}H]LPS-HDL (Figs. 4C–H, 5A). As HDL phospholipids (Fig. 5B) and HDL cholesterol (data not shown) decreased, the fraction of the [^{3}H]LPS that bound to VLDL increased, while the fraction of the [^{3}H]LPS that bound to HDL decreased. Similarly, as HDL decreased, the fraction of the [^{3}H]LPS that bound to LDL increased (Fig. 5C). Less than 50% of the [^{3}H]LPS was found in HDL in almost all of the hypertriglyceridemic samples (Fig. 5A). Thus, VLDL and LDL collectively become the dominant LPS acceptor lipoproteins in hypertriglyceridemic serum.

In keeping with a report that septic patients had increased levels of circulating apoE-HDL (35), we found that some of the lipoprotein-bound [^{3}H]LPS coeluted with apoE-containing particles (presumably apoE-HDL)

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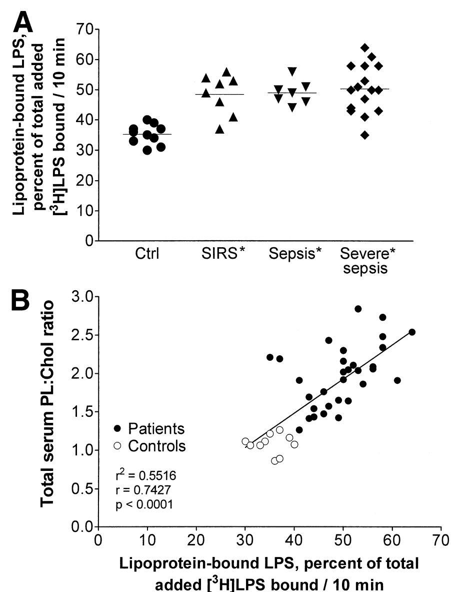

Fig. 3. The rate of lipopolysaccharide (LPS) binding to serum lipoproteins is increased during critical illness. $[^3H]$LPS was incubated with serum for 10 min at 37°C, and the total lipoprotein fraction of the serum was isolated. A: The data (*y* axis) are expressed as the percent of the total added $[^3H]$LPS that bound to lipoproteins per 10 min. Subjects were segregated by healthy controls (Ctrl) and by clinical classification of the patients. The results in each of the three clinical groups were significantly different from the controls * $P < 0.01$. B: The data in A were plotted (*x* axis) against the ratio of phospholipids to total serum cholesterol (*y* axis), and the results were analyzed by linear regression.

that were intermediate in size between HDL and LDL (Fig. 4H and data not shown) in nine of our hypertriglyceridemic patients. The sizes of the apoE-containing particles were clearly larger than those of the particles that contained apoA-I and SAA in six of these patients. These data, taken together with the data shown in Fig. 5, support the hypothesis that in some hypertriglyceridemic patients, almost all of the lipoprotein-bound LPS was associated with particles that contain apoE and/or apoB.

Our results do not rule out the possibility that the high phospholipid content of the VLDL fraction in some septic patients is due in part to the presence of large phospholipid-rich particles that are distinct from VLDL. The VLDL phospholipid-to-apolipoprotein ratios in some septic patients [e.g., 2.9 (Fig. 4G, H)] were somewhat higher than those of controls [e.g., 1.9 (Fig. 1A, B)]. However, the average ratios in each clinical group were not significantly different from the controls (data not shown).

Acute-phase lipoproteins neutralize the bioactivity of LPS

We pooled serum samples from control subjects or patients, incubated them with $[^3H]$LPS, and isolated the $[^3H]$LPS-lipoprotein complexes from the serum. LPS in the lipoprotein complexes had essentially no ability to stimulate human monocytes to release TNF-α (**Fig. 6**) or IL-6 (data not shown). In a similar experiment, $[^3H]$LPS-lipoprotein complexes from pooled hypertriglyceridemic serum samples were isolated by differential ultracentrifugation into VLDL/IDL ($d < 1.019$), LDL ($1.019 < d < 1.063$), and HDL ($1.063 < d < 1.21$) fractions. In the monocyte stimulation assay, the lipoprotein-bound LPS in each density fraction was essentially devoid of bioactivity (data not shown). Our data therefore suggest that inflammation and sepsis-induced changes in plasma may accelerate both the binding and neutralization of LPS by lipoproteins.

DISCUSSION

Our results show that the lipoproteins in human serum can rapidly sequester LPS, even when total cholesterol levels are low during critical illness. Contrary to previous speculation, it thus seems unlikely that the hypocholesterolemia of critical illness impairs the neutralization of circulating LPS by decreasing LPS-lipoprotein binding (8, 10–12). Although much attention has been focused on cholesterol as a measure of lipoprotein levels, our results suggest that in critically ill patients, serum cholesterol is a poor indicator of the capacity of lipoproteins to bind LPS. This may be explained in part by the observation that the loss of cholesterol is due to the loss of esterified cholesterol from the core of lipoprotein particles, whereas little or no material (phospholipids, protein, and free cholesterol) is lost from lipoprotein surface domains where LPS binds (Fig. 1 and data not shown). Phospholipid levels more accurately reflect lipoprotein surface area than do total cholesterol levels, because phospholipids and proteins increase in proportion to the surface area of the particle (15).

We found that the distribution of lipoprotein-bound LPS among the major lipoprotein classes in our patients paralleled the phospholipid content of those classes (Figs. 4, 5). The distribution of phospholipids in the lipoprotein classes may thus explain the patterns of LPS-lipoprotein binding that we observed. HDL has been called a "surface lipoprotein," because most of the mass of the HDL particle is in its surface domain, which is enriched in phospholipids. It has been estimated that in normal plasma, there are at least 10-fold more HDL particles than the combined number of all other lipoprotein particles (21). This may account for the observation that very little LPS binds to VLDL in healthy individuals [even in those with type IV hyperlipidemia (27)]. Therefore, as observed here, HDL may continue to bind the majority of the LPS until HDL levels are critically low and phospholipid levels in the VLDL and LDL fractions are elevated (Fig. 5).

Our analysis of phospholipids in the major lipoprotein classes suggests that a compensatory mechanism may exist to maintain serum phospholipids when HDL levels become critically low. We found that phospholipids were lost only from the HDL fraction (Table 1) and that the lowest HDL levels were accompanied by elevated serum phospholipids, free cholesterol, and triglycerierides. Compensation might also occur by increasing triglycerides in response to the further loss of cholesterol esters, as was seen in some of our severe sepsis patients. In any case, in

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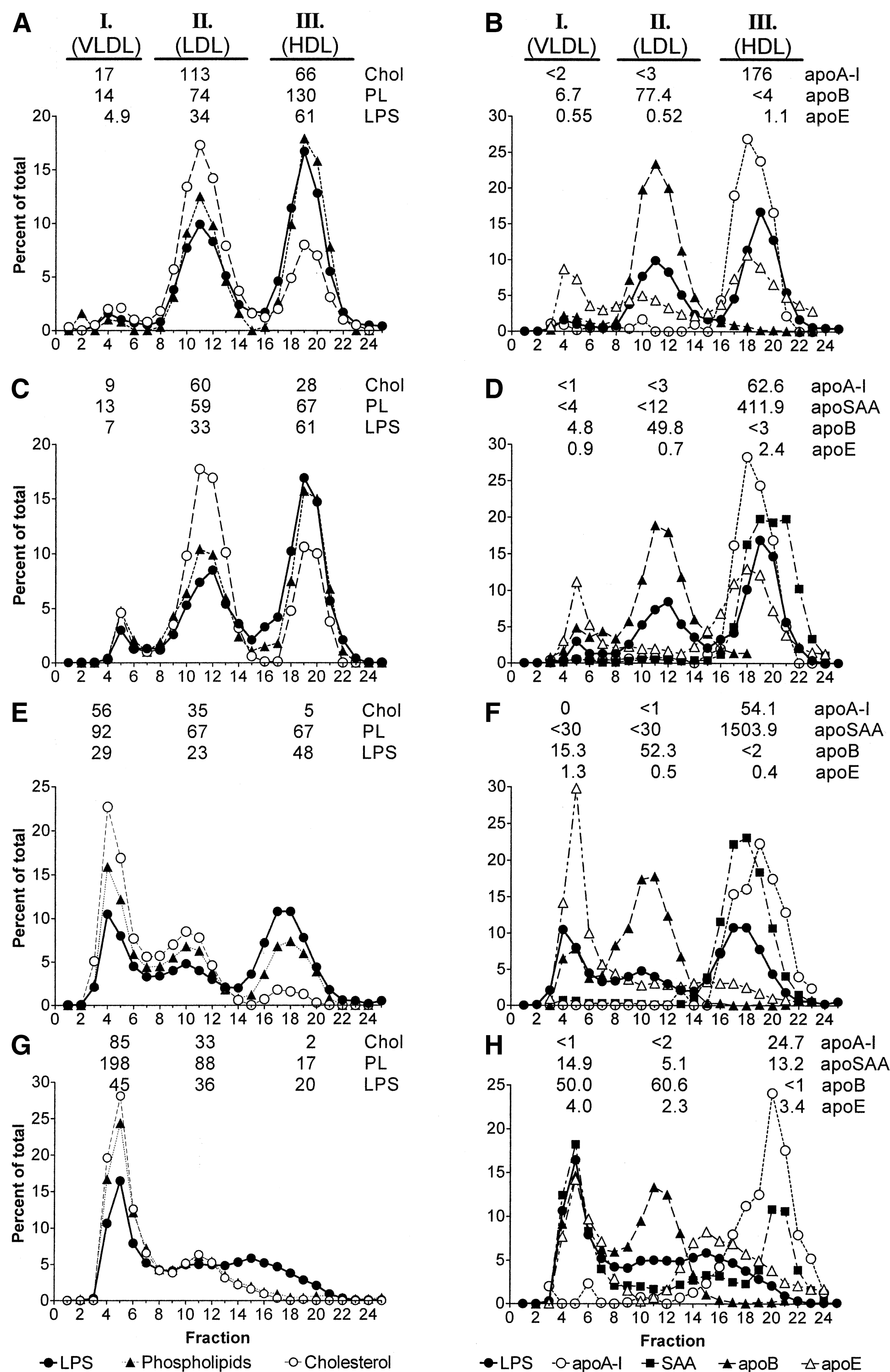

Fig. 4. Chromatographic separation of lipoproteins in representative samples. Serum samples from a healthy control subject (A, B), a patient with sepsis (E, F), and patients with severe sepsis (C, D and G, H) were incubated with [^{3}H]LPS (50 ng/ml) for 10 min, and the total lipoprotein fraction was then isolated by ultracentrifugal flotation. The lipoproteins were then fractionated on a Superose 6 column, and the fractions were assayed for [^{3}H]LPS, total cholesterol, phospholipids (A, C, E, G), and apolipoproteins (B, D, F, H). Serum concentrations (mg/dl) of cholesterol (Chol), phospholipids (PL), and apolipoproteins in each lipoprotein class were determined by multiplying the content of each class (percent of total) by the total concentration of each component in the serum sample. The LPS content of each peak is shown as percent of the total LPS. The values shown in A and B are means of the control subjects.

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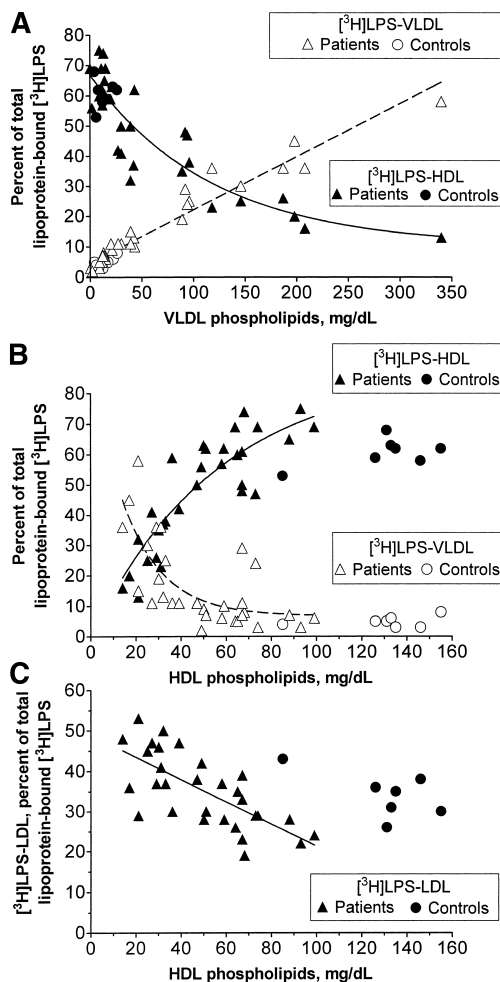

Fig. 5. LPS binding to VLDL and LDL vary inversely with HDL levels. Lipoproteins from controls and critically ill patients were analyzed as described in Fig. 4. A: VLDL phospholipid levels, computed as described in Table 1, were plotted against percentages of the total lipoprotein-bound [^{3}H]LPS found in the HDL peak ([^{3}H]LPS-HDL; $r = -0.8869$) or in the VLDL peak ([^{3}H]LPS-VLDL; $r = 0.9689$; $P < 0.0001$). B: HDL phospholipid levels, also computed as described in Table 1, were plotted against [^{3}H]LPS-HDL ($r = 0.8836$) or [^{3}H]LPS-VLDL ($r = -0.7294$). C: HDL phospholipid levels were plotted against [^{3}H]LPS-LDL ($r = -0.7099$; $P < 0.0001$). Controls were excluded from the regression analyses in B and C.

critically ill patients, lipids are carried principally by LDL and VLDL, and the increased phospholipid content of these fractions corresponds to their increased ability to bind LPS (Figs. 2, 5). Others have reported that whereas total phospholipid levels decline somewhat in critically ill or infected patients (10, 36–38), they do not decline as precipitously as do total cholesterol levels, which are lost from both HDL and LDL fractions. In keeping with our observations, these studies found that the loss of HDL phospholipids accounted for most of the decline in total phospholipids, whereas little decrease occurred in LDL and no phospholipids were lost from VLDL. Also in keeping with our findings, others have reported that elevated serum phospholipids are associated with infection-induced hypertriglyceridemia (13). Moreover, we found that ele-

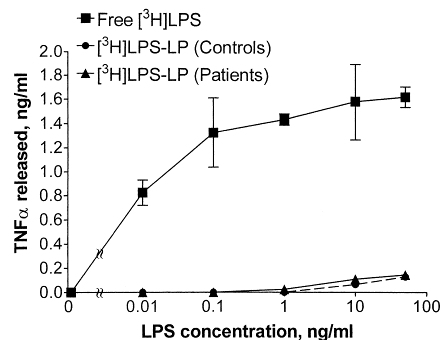

Fig. 6. Acute-phase lipoproteins neutralize the bioactivity of LPS. Normal human monocytes were incubated for 6 h with increasing concentrations of free [^{3}H]LPS or with [^{3}H]LPS-lipoprotein complexes ([^{3}H]LPS-LP) from pooled control subjects or patients. Tumor necrosis factor-α was measured in the culture supernatants. The data are expressed as mean and SD of triplicate wells.

vated triglycerides and phospholipids were also accompanied by increased levels of apoB and apoE (Fig. 4F, H, and data not shown).

The mechanisms that modify lipoproteins during acute illness are multifactoral and are probably driven by bacterial agonists (e.g., LPS) and host cytokines (7, 14, 39). The loss of cholesterol in HDL and LDL may be accounted for largely by a loss of plasma LCAT activity (40), which has been reported to decline to extremely low levels in patients with SIRS and sepsis (35). The decline of LCAT lowers esterified cholesterol in both HDL and LDL but does not decrease phospholipid or free cholesterol levels (40), so other mechanisms must contribute to the decline of HDL phospholipids [e.g., increased activity of secretory phospholipase A_2 ($sPLA_2$) and increased clearance of SAA-HDL] (7, 11, 14, 35). Increased levels of triglycerides and phospholipids are probably driven by the increased availability of free fatty acids derived from peripheral lipolysis, increased de novo hepatic fatty acid synthesis, and decreased hepatic fatty acid oxidation. Decreased lipoprotein lipase activity may also increase circulating triglycerides (7, 14, 39).

Although the LPS-binding capacity of plasma lipoproteins greatly exceeds the amount of circulating LPS (24, 41), lipoproteins decrease (1) but do not entirely prevent LPS responses in cells that are exposed to the blood (e.g., circulating monocytes, endothelial cells, and Kupffer cells). We have shown that the ability of circulating monocytes to respond to LPS can be explained by the observation that LPS binds to monocytes more rapidly than it binds to lipoproteins (23, 29). Thus, monocyte responses to LPS are likely to be influenced more by the rate of LPS binding to lipoproteins than by the total capacity of lipoproteins to bind LPS. During critical illness, LPS-monocyte interactions may be reduced in two general ways: *i*) acute-phase lipoproteins may compete with monocytes and other LPS-responsive cells more effectively for binding LPS, and *ii*) lipoproteins and soluble CD14 accelerate the removal of LPS from monocyte surfaces and thereby attenuate responses to the LPS that has already bound to

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them (29). We previously showed that the latter mechanism is enhanced in the serum of injured and septic patients (29), and our data now suggest that the first mechanism is also more active under the same clinical conditions. It should be noted, however, that the ex vivo whole-blood studies of Gordon and coworkers (25) suggest that adding reconstituted lipoproteins to the blood of critically ill patients could have an additional antiinflammatory effect, presumably by increasing the rate of LPS binding to the lipoprotein fraction of plasma.

The mechanism that accelerates the binding of free LPS to acute-phase lipoproteins is unclear. Whereas LBP may be important for transferring LPS to lipoproteins (22), in this study we found that variations in serum LBP levels did not correlate with variations in the rate of LPS binding to lipoproteins, suggesting that LPS-lipoprotein binding is not modulated by changes in the concentration of LBP. However, we cannot exclude the possibility that phospholipid transfer protein, which is also increased in the blood of septic patients (35), might enhance LPS binding to lipoproteins (22). Changes in the composition of plasma lipoproteins during inflammation and infection (7, 39) might also influence LPS-lipoprotein binding. Our data (Table 1) show that the ratio of phospholipids to cholesterol was usually increased above control levels in all lipoprotein classes and in all clinical groups and that this ratio correlated with the rate of LPS binding to lipoproteins (Fig. 3B). This correlation suggests that phospholipid enrichment may contribute to the enhanced ability of the modified lipoproteins to bind LPS. However, we cannot rule out the possibility that the phospholipid-to-cholesterol ratio correlates with another serum alteration that is mechanistically important. The positive correlation between total serum apoE and LPS-lipoprotein binding ($r = 0.63$; $P < 0.0001$) raises the possibility that apoE may play a role. This hypothesis is supported by the known ability of apoE to bind LPS (42) and by our findings that significant positive correlations exist between the percentages of the total lipoprotein-bound LPS and the percentages of the total apoE found in each respective lipoprotein class (LPS-HDL vs. apoE-HDL: $r = 0.72$, $P < 0.0001$; LPS-LDL vs. apoE-LDL: $r = 0.41$, $P = 0.014$; LPS-VLDL vs. apoE-VLDL: $r = 0.66$, $P < 0.0001$).

We cannot rule out the possibility that structural differences among LPSs derived from different bacterial strains or species might influence the binding of the LPS to different lipoprotein classes. However, the distribution of our [^{3}H]LPS among the major lipoprotein classes generally agrees with that of Levels and coworkers (24), who studied, using a fluorescence dequenching method, the binding of three widely different forms of LPS (O111:B4 and J5 from *E. coli* and Re595 from *Salmonella typhimurium*) to lipoproteins in normal human serum. Whereas their data suggest that some of the HDL-bound LPS moved to LDL or VLDL between short (10 min) and long (60 min) incubation times, we found that the distribution of our [^{3}H]LPS among the major lipoprotein classes was identical at both of these time points (data not shown).

Although the extent to which apolipoproteins influence LPS-lipoprotein binding is unclear, they are important determinants of the clearance of LPS-lipoprotein complexes. SAA enhances the clearance of HDL [as reviewed in ref. (11)], and the association of LPS with apoE- and apoB-containing particles may greatly increase LPS clearance by promoting its uptake by receptors of the LDL receptor family. Whereas LPS that is complexed with normal HDL circulates for many hours (2), LPS that is complexed with apoE-containing CM remnants (β-VLDL) is cleared within minutes (43), principally by hepatocytes. Because LPS-lipoprotein complexes that are isolated from serum by density flotation are essentially devoid of bioactivity (Fig. 6), the physiological importance of their rapid in vivo clearance is uncertain. However, it should be noted that a substantial fraction of the LPS that is added to serum does not become irreversibly bound to lipoproteins (see Results and Fig. 3A); a portion of this LPS might bind to proteins that are loosely associated with lipoproteins [e.g., LBP (44) and $sPLA_2$ (45, 46)] and might still be able to stimulate LPS-sensitive cells. Rapid clearance of this kind of LPS-lipoprotein complex may have greater functional importance than the clearance of tightly associated LPS-lipoprotein complexes. LPS complexes with apoE-containing lipoproteins may also have antiinflammatory properties. For example, apoE can redirect LPS from Kupffer cells to hepatocytes (42), and Harris and coworkers (47) have reported that apoE-containing LPS-lipoprotein complexes can downregulate inflammatory responses to cytokines in primary hepatocytes by a mechanism that is mediated by the LDL receptor.

In recent years, infection- and inflammation-induced changes in circulating lipoproteins have attracted interest because of their presumed proinflammatory and proatherogenic properties (7). Our results suggest that with regard to host defense against bacterial infection, these changes may actually help reduce inflammatory reactions.

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